Journal of Insect Science
www.insectscience.org

Analysis of genetic variation and phylogeny of the predatory bug, *Pilophorus typicus*, in Japan using mitochondrial gene sequences

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Abstract

Pilophorus typicus (Distant) (Heteroptera: Miridae) is a predatory bug occurring in East, Southeast, and South Asia. Because the active stages of *P. typicus* prey on various agricultural pest insects and mites, this species is a candidate insect as an indigenous natural enemy for use in biological control programs. However, the mass releasing of introduced natural enemies into agricultural fields may incur the risk of affecting the genetic integrity of species through hybridization with a local population. To clarify the genetic characteristics of the Japanese populations of *P. typicus* two portions of the mitochondrial DNA, the cytochrome oxidase subunit I (*COI*) (534 bp) and the cytochrome B (*cytB*) (217 bp) genes, were sequenced for 64 individuals collected from 55 localities in a wide range of Japan. Totals of 18 and 10 haplotypes were identified for the *COI* and *cytB* sequences, respectively (25 haplotypes over regions). Phylogenetic analysis using the maximum likelihood method revealed the existence of two genetically distinct groups in *P. typicus* in Japan. These groups were distributed in different geographic ranges: one occurred mainly from the Pacific coastal areas of the Kii Peninsula, the Shikoku Island, and the Ryukyu Islands; whereas the other occurred from the northern Kyushu district to the Kanto and Hokuriku districts of mainland Japan. However, both haplotypes were found in a single locality of the southern coast of the Shikoku Island. *COI* phylogeny incorporating other *Pilophorus* species revealed that these groups were only recently differentiated. Therefore, use of a certain population of *P. typicus* across its distribution range should be done with caution because genetic hybridization may occur.

Keywords: biological control; cytochrome B (*cytB*); cytochrome oxidase subunit I (*COI*); indigenous natural enemy; phylogenetic analysis
Abbreviations: ***COI***, cytochrome oxidase subunit I; **cytB**, cytochrome B; **ML,** maximum likelihood; **NJ,** neighbor-joining; **TBR**, tree-bisection-reconnection
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Editor: Mariana Wolfner was editor of this paper.
Received: 1 December 2009, **Accepted:** 7 May 2010

ISSN: 1536-2442 | Vol. 11, Number 18

Cite this paper as:
Ito K, Nishikawa H, Shimada T, Ogawa K, Minamiya Y, Tomoda M, Nakahira K, Kodama R, Fukuda T, Arakawa R. 2011. Analysis of genetic variation and phylogeny of the predatory bug, *Pilophorus typicus,* in Japan using mitochondrial gene sequences. *Journal of Insect Science* 11:18 available online: insectscience.org/11.18

Introduction

Introducing natural enemies as control agents for agricultural pests has long been attempted in hope of long lasting suppression effects, reducing pesticide chemicals, saving labor, cutting costs, etc. However, introducing an alien natural enemy into a new agroecosystem may incur ecological and genetic risks. Ecologically, they may secondarily attack non-target insects and drive them into extinction (reviewed in Howarth 1991; Simberloff and Stiling 1996). Genetically, the mass release of introduced natural enemies into agricultural fields may affect the genetic integrity of a local population of species through hybridization. To avoid these risks, utilization of indigenous natural enemies, i.e. mass-reared predators collected from the local area, has been attempted by release into agricultural fields because their ecology and genetic background may be similar to the local population as compared to one that is exotic, and thus may more easily adapt to the local environment with fewer risks. However, geographic proximity does not necessarily reflect genetic distance. For example, a recent phylogenetic study showed that close local populations of a parasitic wasp that were used as a natural enemy was composed of multiple cryptic strains that were different in host use and other life histories (Phillips et al. 2008). Thus, phylogenetic analyses can provide primary data of genetic structure of an indigenous natural enemy, allowing inference about ecological and genetic consequences in the application field.

Pilophorus typicus (Distant 1909) (Heteroptera: Miridae) is a candidate as an indigenous natural enemy in biological control programs in Japan. This is polyphagous predatory bug that looks like an ant (Ito et al. 2010). This species occurs in Japan, Taiwan, China, the Philippines, Indochina, Malaysia, Indonesia, Sri Lanka, and India (Schuh 1984). In Japan, this species is distributed from the Ryukyu Islands to Honshu of the mainland (Yasunaga 2001). Adults (approximately 2.7 mm long) and larvae are usually found on various wild plants and greenhouse crops (Yasunaga 2001). Because *P. typicus* preys on various agricultural pests such as whiteflies, thrips, and spider mites (H. Nishikawa et al. unpublished data) that damage commercially important vegetables such as tomato, eggplant, and green pepper under greenhouse conditions. However, degrees of genetic differentiation among geographic populations of *P. typicus* are presently completely unknown.

In various insect groups, nucleotide sequence information of several gene regions on mitochondrial DNA (mtDNA) has been used for evaluating phylogenetic relationships among closely related species or genetically heterogeneous populations of a single species because these regions show sufficiently high rates of nucleotide substitution (e.g. Hebert et al. 2003; Pons et al. 2004; Havill et al. 2007). In particular, the cytochrome oxidase subunit I (*COI*) has been most frequently used in phylogenetic analyses (Hebert et al. 2003), or studies of the genetic structure of agricultural pests (Smith 2005). The cytochrome B (*cytB*) gene has been proved to have the same level of sequence variation as the *COI* region for phylogenetic analysis of many insect orders (Simmons and Weller 2001), and though used less frequently than *COI*, this region has been used for phylogenetic analyses in Heteroptera (e.g. Muraji et al. 2000a, 2000b, 2001). In this study, partial regions of the *COI* and *cytB* genes of *P. typicus* specimens collected from a wide range of Japan were sequenced, and

attributes of sequence variation in each region as well as phylogenetic relationship within *P. typicus* using combined sequences were investigated. In addition, the degree of the sequence variation was compared with that found between other *Pilophorus* species to infer the taxonomic status of the phylogenetic groups.

Materials and Methods

Mites

Sixty-four individuals of *P. typicus* sampled from 55 localities covering the Ryukyu Islands and the Japanese mainland from Kyushu to Honshu were used for the analysis of the *COI* and *cytB* sequences (Table 1). One individual was analyzed for 47 localities, two for 7 localities, and three for 1 locality. One individual *P. setulosus* collected in the Hokuriku district was sequenced and used as an outgroup. All sample individuals were stored at -30° C until DNA extraction.

PCR and sequencing procedure

The whole body of a sample individual was ground with a plastic pestle in a 1.5 ml microcentrifuge tube containing 200 µl of HMW buffer (10 mM Tris, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA)-2Na (pH 8.0), 1.255% (w/v) sodium dodecylsulfate (SDS) and 0.1 mg/ml proteinase K). After incubation of the mixture at 55° C for 30 min, 500 µl phenol-saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8) were added and mixed thoroughly. The mixture was centrifuged at 14,000 rpm for 10 min at 4° C to separate phases. The upper aqueous phase was mixed with 500 µl of chloroform:isoamyl alcohol (24:1) mixture and centrifuged. The upper phase was dissolved in 500 µl of 100% ethanol with 20 µl of 3M sodium acetate to precipitate DNA. The precipitate was collected by centrifugation, washed with 120 µl of 70 % ethanol, partially dried under the vacuum, and then resuspended in 30 µl of TE buffer. DNA samples were stored at -20° C until use.

PCR was performed in a 50 µl reaction mixture containing 1.25 µl of DNA sample, 1 X PCR buffer (10 mM Tris-HCl buffer (pH 8.3 at 25° C), 50 mM KCl, and 1.5 mM $MgCl_2$); 0.16 mM of each dNTP, 0.3 mM of each primer, and 1.25 U of rTaq DNA polymerase (TOYOBO). After incubation at 94° C for 30 sec, DNA was amplified by 45 cycles of incubation at 94° C for 1 min, 48° C for 2 min, and 72° C for 2 min with a final extension at 72° C for 15 min. The *COI* region was amplified using primers previously reported by Folmer et al. (1994): LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3') and HCO2198 (5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3'). The *cytB* region was amplified using degenerate primers manually designed from the consensus sequence of the partial *cytB* regions of Miridae and related species deposited in DDBJ/EMBL/GenBank DNA databases (EU401991, AY327435, AY327430, AY916050, DQ372123): *cytB*-F1_10623 (5'-ATT AC(A/T) AAT (T/C)TA CT(A/C) TCA GC-3') and *cytB*-R1_11002 (5'-CAT TCT GGT TG(A/G) ATG TG(G/T) AC-3'). Attached numbers agree with the annealing positions in reference to the mitochondrial genome of *Lygus lineolaris* (EU401991). After amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gels (Agarose-L, NipponGene), and DNA bands stained with ethidium bromide were excised and purified with QIAquick Gel Extraction Kit (Qiagen, www.qiagen.com). Sequence analyses were conducted using a BigDye

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, www.appliedbiosystems.com) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to manufacturer's instructions. Sequence primers were the same as used in

Table 1. Summary of *Pilophorus* specimens analyzed in this study. Details of haplotypes are summarized in Table 2

		Locality				Sampling date			COI	CytB
	Population	District	Prefecture	City	Town		COI	CytB	Accession No.	Accession No.
Ingroup species, *Pilophorus typicus*										
1	Iriomote13	Ryukyu	Okinawa	Taketomi	Toyohara	18 Nov, 2006	1	1	AB439610	AB439675
2	Iriomote1	Ryukyu	Okinawa	Taketomi	Toyohara	18 Nov, 2006	1	3	AB439608	AB439673
3	Iriomote10	Ryukyu	Okinawa	Taketomi	Toyohara	18 Nov, 2006	1	4	AB439609	AB439674
4	Ishigaki	Ryukyu	Okinawa	Ishigaki	Kabira	22 Nov, 2006	2	1	AB439607	AB439672
5	Okinawa	Ryukyu	Okinawa	Naha	Shurisakiyamada	Jun, 2006	1	1	AB439606	AB439671
6	Shibushi	Kyushu	Kagoshima	Shibushi	Shibushi	21 Nov, 2007	1	5	AB439605	AB439670
7	Kagoshima	Kyushu	Kagoshima	Kagoshima	Korimoto	17 Sep, 2006	1	1	AB439603	AB439668
8	Hioki	Kyushu	Kagoshima	Hioki	Fukiagecho	20 Nov, 2007	1	1	AB439604	AB439669
9	Kashiwajima	Shikoku	Kochi	Otsuki	Kashiwajima	24 Aug, 2007	1	2	AB439596	AB439661
10	Odokaigan	Shikoku	Kochi	Otsuki	Odokaigan	24 Aug, 2007	1	1	AB439600	AB439665
11	Sukumo85	Shikoku	Kochi	Sukumo		24 Aug, 2007	1	1	AB439601	AB439666
12	Sukumo86	Shikoku	Kochi	Sukumo		24 Aug, 2007	3	1	AB439602	AB439667
13	Shiwa	Shikoku	Kochi	Shimanto	Shiwa	24 Aug, 2007	1	1	AB439599	AB439664
14	Muroto7	Shikoku	Kochi	Muroto	Murotomisaki	30 Jun, 2007	1	1	AB439598	AB439663
15	Otake	Chugoku	Hiroshima	Otake	Misono	9 Aug, 2007	1	2	AB439594	AB439659
16	Takehara	Chugoku	Hiroshima	Takehara	Takasaki	9 Aug, 2007	1	2	AB439595	AB439660
17	Shionomisaki14	Kinki	Wakayama	Kushimoto	Shionomisaki	9 Jul, 2007	1	2	AB439593	AB439658
18	Shionomisaki13	Kinki	Wakayama	Kushimoto	Shionomisaki	9 Jul, 2007	4	2	AB439592	AB439657
19	Odomari	Kinki	Wakayama	Kumano	Odomari	9 Jul, 2007	5	1	AB439597	AB439662
20	Uki	Kyushu	Kumamoto	Uki	Matsubasemachi	20 Nov, 2007	8	7	AB439635	AB439700
21	Saiki	Kyushu	Oita	Saiki	Tsurumi	22 Jul, 2007	6	6	AB439655	AB439720
22	Kunisaki	Kyushu	Oita	Kunisaki	Kunimi	8 Aug, 2007	6	7	AB439634	AB439699
23	Kanda	Kyushu	Fukuoka	Kanda	Yobaru	8 Aug, 2007	6	6	AB439653	AB439718
24	Munakata	Kyushu	Fukuoka	Munakata	Mochiyama	19 Sep, 2007	6	6	AB439654	AB439719
25	Narihata	Shikoku	Kochi	Otsuki	Narihata	24 Aug, 2007	6	7	AB439633	AB439698
26	Kubokawa	Shikoku	Kochi	Takaoka	Shimanto	24 Aug, 2007	6	6	AB439651	AB439716
27	Muroto6	Shikoku	Kochi	Muroto	Murotomisaki	30 Jun, 2007	6	6	AB439652	AB439717
28	Yoshidacho	Shikoku	Ehime	Yoshida	Chinaga	22 Jul, 2007	6	7	AB439631	AB439696
29	Iyo	Shikoku	Ehime	Iyo	Miaki	22 Jul, 2007	9	6	AB439650	AB439715
30	Imabari	Shikoku	Ehime	Imabari	Niya	22 Jul, 2007	10	6	AB439649	AB439714
31	Shodoshima	Shikoku	Kagawa	Shodo	Shodoshima	26 Aug, 2007	6	7	AB439632	AB439697
32	Shimonoseki70	Chugoku	Yamaguchi	Shimonoseki	Toyoura	7 Aug, 2007	6	7	AB439630	AB439695
33	Shimonoseki69	Chugoku	Yamaguchi	Shimonoseki	Toyoura	7 Aug, 2007	11	7	AB439629	AB439694
34	Tokuyama	Chugoku	Yamaguchi	Tokuyama	Sakaedani	8 Aug, 2007	6	6	AB439648	AB439713
35	Hagi	Chugoku	Yamaguchi	Hagi	Oi	7 Aug, 2007	6	6	AB439647	AB439712
36	Fukuyama	Chugoku	Hiroshima	Fukuyama	Zao	9 Aug, 2007	12	8	AB439642	AB439707
37	Sugano	Chugoku	Okayama	Okayama	Sugano	6 Aug, 2007	13	6	AB439641	AB439706
38	Maniwa	Chugoku	Okayama	Maniwa	MimasakaOiwake	6 Aug, 2007	6	9	AB439628	AB439693
39	Hamada63	Chugoku	Shimane	Hamada	Misumi	7 Aug, 2007	6	6	AB439644	AB439709
40	Hamada64	Chugoku	Shimane	Hamada	Misumi	7 Aug, 2007	14	6	AB439645	AB439710
41	Ota	Chugoku	Shimane	Ota	Asayama	7 Aug, 2007	15	6	AB439646	AB439711
42	Yonago	Chugoku	Tottori	Yonago		6 Aug, 2007	6	10	AB439643	AB439708
43	Gobo	Kinki	Wakayama	Gobo	Noguchi	9 Jul, 2007	16	6	AB439639	AB439704
44	Kainan	Kinki	Wakayama	Kainan	Shimotsu	9 Jul, 2007	6	6	AB439638	AB439703
45	Minamiise	Kinki	Mie	Minamiise		10 Jul, 2007	6	7	AB439626	AB439691
46	Tsu	Kinki	Mie	Tsu	Edobashi	10 Jul, 2007	6	6	AB439640	AB439705
47	Suzuka	Kinki	Mie	Suzuka	Jike	10 Jul, 2007	6	7	AB439627	AB439692
48	Morozaki	Chubu	Aichi	Minamichita	Morozaki	10 Jul, 2007	6	7	AB439625	AB439690
49	AichiMito	Chubu	Aichi	Mito	Akane	11 Jul, 2007	7	6	AB439611	AB439676
50	Hamamatsu	Chubu	Shizuoka	Hamamatsu	Matsushima	11 Jul, 2007	7	6	AB439613	AB439678
51	Abegawa	Chubu	Shizuoka	Suruga	Abegawa	11 Jul, 2007	6	6	AB439612	AB439677
52	Mishima185	Chubu	Shizuoka	Mishima	Kawaharagaya	11 Jul, 2007	6	7	AB439636	AB439701
53	Mishima186	Chubu	Shizuoka	Mishima	Kawaharagaya	11 Jul, 2007	7	6	AB439637	AB439702
54	Tsukuba	Kanto	Ibaraki	Tsukuba	Nishioka	July, 2006	6	7	AB439624	AB439689
55	Katsuyama	Hokuriku	Fukui	Katsuyama		10 Sep, 2007	7	6	AB439616	AB439681
56	Fukui116	Hokuriku	Fukui	Fukui		10 Sep, 2007	7	6	AB439615	AB439680
57	Fukui114	Hokuriku	Fukui	Fukui		10 Sep, 2007	17	6	AB439614	AB439679
58	Tedorigawa	Hokuriku	Ishikawa	Hakusan	Mikawa	13 Sep, 2007	18	6	AB439617	AB439682
59	Ikeda	Hokuriku	Toyama	Toyama	Ikeda	11 Sep, 2007	7	6	AB439618	AB439683
60	Kurobe	Hokuriku	Toyama	Kurobe	Unazuki	11 Sep, 2007	7	6	AB439620	AB439685
61	Kamitaki	Hokuriku	Toyama	Toyama	Kamitaki	11 Sep, 2007	7	6	AB439621	AB439686
62	Tateyama	Hokuriku	Toyama	Tateyama		11 Sep, 2007	7	6	AB439622	AB439687
63	Furudo	Hokuriku	Toyama	Toyama		11 Sep, 2007	7	6	AB439619	AB439684
64	Hiraiwa	Hokuriku	Nigata	Itoigawa	Hiraiwa	11 Sep, 2007	7	6	AB439623	AB439688
Outgroup species, *Pilophorus setulosus*										
	Kurobe	Hokuriku	Toyama	Kurobe	Unazuki	11 Sep, 2007	19	11	AB439656	AB439721

PCR reaction.

Sequence data were aligned using Clustal W 1.83 (Thompson et al. 1994) with default parameter setting. To evaluate data, nucleotide compositions in each codon position, proportions of variable sites, and transition/transversion rates were investigated for each region using MEGA4 software (Tamura et al. 2007). The degree of saturation was assessed for each region by pairwise plotting of the proportion of different sites between two sequences at each codon position against the Tamura-Nei distance (Tamura and Nei 1993) between them including all codon positions. Moreover, genetic divergence within phylogenetic groups was estimated using the number of base substitutions per site from averaging over all sequence pairs within each group (Nei and Kumar 2000). The analyses were conducted using the Tamura-Nei method in MEGA4. To assess the congruence of the two regions, the partition homogeneity test (Farris et al. 1994, 1995) was conducted using the HOMPART command (1000 replicates) implemented on the software PAUP* ver. 4.0b10 (Swofford 2003).

Phylogenetic analysis of *P. typicus*

As a preliminary test, the phylogenetic analysis based on the neighbor-joining (NJ) method was performed separately for the *COI* and *cytB* regions using MEGA4 to investigate the degree of consistency of mutation patterns in different regions. In these analyses, the nucleotide substitution model for each region was selected using the likelihood ratio test with the program Modeltest 3.7 (Posada and Crandall 1998). Reliability of branches was estimated by 1000 bootstrap resamplings.

The combined sequences were subjected to the phylogenetic analysis of the maximum likelihood (ML) method using the heuristic search algorithm through the HSEARCH command in PAUP*. The selection of the nucleotide substitution model and the estimation of the substitution rate matrix were conducted on Modeltest. The starting tree was obtained via the neighbor-joining method, and used for the heuristic search of the ML tree by tree-bisection-reconnection (TBR) swapping (HSEARCH command: criterion = likelihood, addseq = random, nreps = 10). Other parameters were set according to default values in the HSEARCH command. The reliability of internal branches was assessed by 1000 bootstrap resamplings with TBR and the same parameter set as used in constructing the original ML tree.

Variation within *Pilophorus*

To understand the degree of observed genetic variation in the light of intrageneric variation, the phylogeny of the *COI* region including other *Pilophorus* species was investigated. In addition to three newly obtained sequences of *P. typicus* (Muroto6 and Muroto7) and *P. setulosus*, the sequences of four other species and one unidentified strain of *Pilophorus* (DDBJ/EMBL/GenBank: AY252988, AY253015, AY253025, AY253083, AY253102) were used for the analysis. The tree was rooted with the sequence (EU427341) of an anthocorid bug *Orius niger* (Wolff) (Hemiptera: Anthocoridae), whose life history is similar to *Pilophorus* bugs. ML analysis was conducted using homologous 533 bp as in the above analyses. The *cytB* sequences were not analyzed because of the scarcity of sequence information in *Pilophorus*.

Results and Discussion

The aligned sequence lengths of the *COI* and *cytB* regions analyzed were 534 and 217 bp,

Table 2. Sequence variation in the *COI* and *cytB* regions of the mitochondrial DNA of *Pilophorus typicus* in Japan. Dots indicate identity with the consensus sequence. Clade names agree with those in Fig. 3.

Haplotype	OTU label	n	Variable sites (COI 534 bp)																																				Accession number
			1	5	11	56	57	64	71	79	128	136	140	164	209	230	275	301	323	326	344	363	372	380	398	399	403	404	456	467	498	509	516	525					
			1	5	11	56	57	64	71	79	128	136	140	164	209	230	275	301	323	326	344	363	372	380	398	399	403	404	456	467	498	509	516	525					
Codon position			2	3	3	3	1	2	3	2	3	2	3	3	3	3	3	2	3	3	3	1	1	3	3	1	2	3	1	3	1	3	1	1					
	Consensus		T	A	A	G	C	T	T	T	T	T	T	G	T	T	C	C	T	A	C	G	G	T	A	G	G	A	A	C	G	G	T	C					
Clade I																																							
1	Iriomote1	15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	AB439608				
2	Ishigaki	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	AB439607				
3	Sukumo86	1	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	AB439602				
4	Shionomisaki13	1	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	AB439592				
5	Odomari	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	C	G	.	.	.	.	.	.	.	.	.	AB439597				
Clade II																																							
6	Tsukuba	23	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	.	.	T	.	A	C	T	AB439624				
7	Kurobe	11	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	C	.	T	.	A	C	T	AB439620				
8	Uki	1	.	.	.	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	T	.	.	T	.	A	C	T	AB439635				
9	Iyo	1	.	.	G	A	.	C	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	.	.	T	.	A	C	T	AB439650				
10	Imabari	1	.	T	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	.	.	T	.	A	C	T	AB439649				
11	Shimonoseki69	1	.	.	G	A	.	.	G	.	C	G	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	.	.	T	.	A	C	T	AB439629				
12	Fukuyama	1	.	.	G	A	.	.	G	.	C	.	C	A	C	C	.	T	C	.	T	.	.	.	.	A	.	.	.	T	.	A	C	T	AB439642				
13	Sugano	1	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	.	.	.	.	T	.	A	C	T	AB439641				
14	Hamada64	1	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	T	T	.	.	.	.	A	.	.	.	T	.	A	C	T	AB439645				
15	Ota	1	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	A	.	.	.	A	.	.	.	T	.	A	C	T	AB439646				
16	Gobo	1	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	.	.	T	A	A	C	T	AB439639				
17	Fukui114	1	.	.	G	A	.	.	G	.	C	.	C	A	.	C	.	.	.	.	T	.	.	.	.	A	.	C	.	T	.	A	C	T	AB439614				
18	Tedorigawa	1	.	.	G	A	.	.	A	.	C	.	C	A	.	C	.	.	C	.	T	.	.	.	.	A	.	C	.	T	.	A	C	T	AB439617				
	(Total)																																						

Haplotype	OTU label	n	Variable sites (cytB 217 bp)																Accession number
			3	11	27	50	68	86	121	164	170	176	192	193	206	214			
Codon position			2	1	2	1	1	1	3	1	1	1	2	3	1	3			
	Consensus		A	A	C	C	G	G	C	C	G	C	T	G	T	T			
Clade I																			
1	Ishigaki	11	.	.	.	.	.	.	.	.	.	.	.	.	.	.			AB439672
2	Shionomisaki13	5	.	.	.	.	.	.	T	.	.	.	.	.	.	.			AB439657
3	Iriomote1	1	.	.	T	.	.	.	.	.	.	.	.	.	.	.			AB439673
4	Iriomote10	1	.	.	.	.	.	.	.	A	.	.	.	.	.	.			AB439674
5	Shibushi	1	.	G	.	.	A	A	.	.	.	.	.	.	.	.			AB439670
Clade II																			
6	Kurobe	30	.	.	.	.	.	.	.	.	A	T	C	A	.	.			AB439685
7	Tsukuba	12	.	.	.	.	.	.	.	.	A	T	C	A	.	C			AB439689
8	Fukuyama	1	.	.	.	.	.	.	.	.	A	T	C	A	C	.			AB439707
9	Maniwa	1	G	.	.	.	.	.	.	.	A	T	C	A	.	C			AB439693
10	Yonago	1	.	.	.	A	.	.	.	.	A	T	C	A	.	.			AB439708
	(Total)																		

n indicates the number of identical haplotypes from different samples (see Table 1)

respectively. No insertion or deletion was found in either region. Eighteen haplotypes were detected in the *COI* region, and 10 in the *cytB* region among the 64 individuals of *P. typicus* (Table 2). All sequences have been deposited in DDBJ/EMBL/GenBank DNA databases (Accession numbers: AB439592 and AB439721).

Table 3. Nucleotide composition in the partial *COI* and *cytB* regions of the mitochondrial DNA of *Pilophorus typicus* in Japan

Region	Base	Codon position							
COI		**Overall**		**1st**		**2nd**		**3rd**	
No. sites		534		178		178		178	
No. variable		32	(6.0%)	8	(4.5%)	6	(3.4%)	18	(10.1%)
Nucleotide	T	33.1	(32.8-33.7)	23.6	(23.6 - 24.2)	43.8	(42.7 - 44.4)	32.0	(30.9 - 33.1)
frequency (%)	C	17.2	(16.7-17.4)	15.7	(15.2 - 15.7)	23.6	(23.0 - 24.2)	12	(11.2 - 12.9)
(range)	A	33.1	(32.6-33.5)	34.1	(33.1 - 34.8)	12.4	(12.4 - 12.4)	53	(51.7 - 53.4)
	G	16.6	(16.3-17.2)	26.6	(25.8 - 27.5)	20.2	(19.7 - 21.3)	3.0	(2.2 - 3.9)
Identical pairs*			527		177		178		173
Transitional pairs (si)*			6		1		0		4
Transversional pairs (sv)*			1		0		0		1
si/sv			6.3		-		0.5		5.4
CytB									
No. sites		217		72		72		73	
No. variable		14	(6.5%)	8	(11.1%)	3	(4.2%)	3	(4.1%)
Nucleotide	T	36.3	(35.9 - 36.9)	24.6	(23.6 - 25.0)	47.6	(47.2 - 50.0)	36.8	(35.6 - 38.4)
frequency (%)	C	18.5	(18.0 - 18.9)	24.0	(22.2 - 25.0)	17.6	(15.3 - 18.1)	13.9	(12.3 - 15.1)
(range)	A	36.6	(35.9 - 37.3)	35.7	(34.7 - 37.5)	26.4	(25.0 - 26.4)	47.5	(46.6 - 47.9)
	G	8.6	(8.3 - 9.2)	15.6	(15.3 - 16.7)	8.4	(8.3 - 9.7)	1.8	(1.4 - 2.7)
Identical pairs*			215		71		72		72
Transitional pairs (si)*			2		1		0		1
Transversional pairs (sv)*			0		0		0		0
si/sv			37.7		15.6		-		-

*All frequencies are averages over all taxa rounded to the nearest whole number

The attributes of nucleotide sequences are summarized in Table 3. The partial *COI* and *cytB* regions exhibited a similar proportion of variable sites (about 6% for each). The most variable codon position was 3rd for the *COI* region and 1st for the *cytB* region. Saturation tests plotting the proportion of different sites against the evolutionary distance showed no clear tendency for saturation at either position of each region (results not shown). As shown in Figure 1, the evolutionary rate appears to be slightly higher in the *COI* region when two sequences from evolutionary distant populations were compared. Within 177 and 72 amino acid residues each translated from the *COI* and *cytB* nucleotide sequences, variability was observed at 10 (5.6%) and 9 (12.5%) sites, respectively. The transition and transversion rate (si/sv) was high (6.3 and 37.7, respectively). The partition homogeneity test on PAUP showed no significant incongruence between the two regions (P = 1.000).

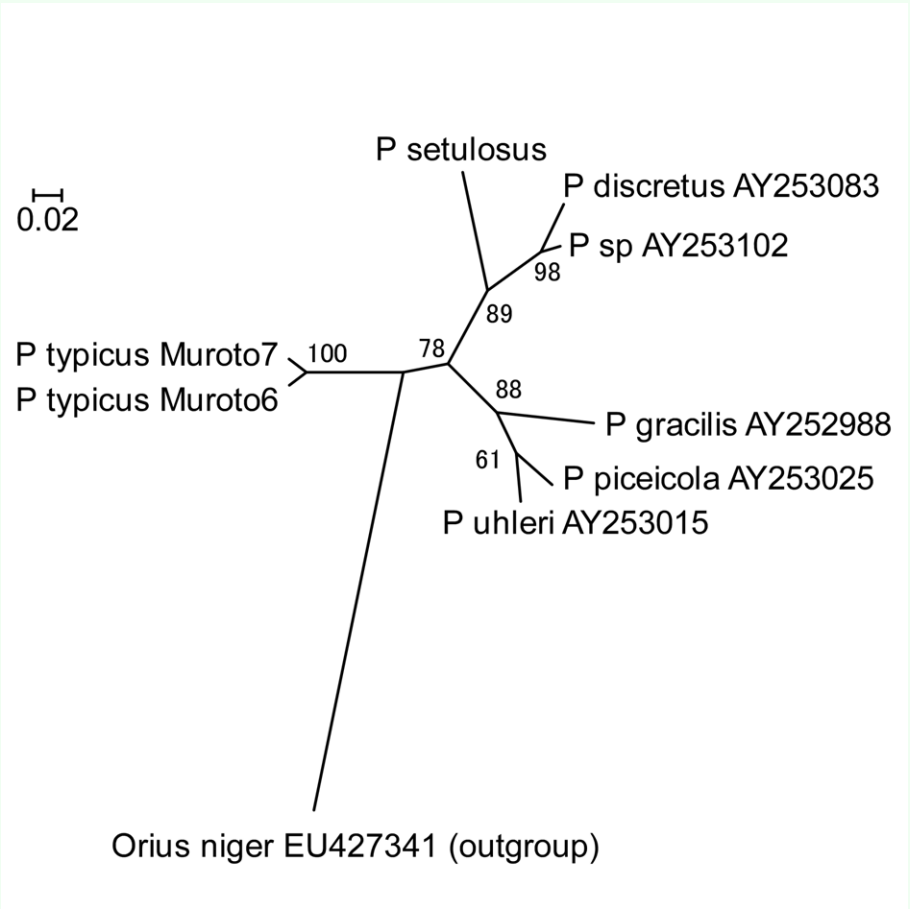

Figure 1. Relationship between Tamura-Nei distances of *COI* and *cytB* regions for each sample. All codon positions are included. Line indicates the set of points on which the distances are equal. High quality figures are available online.

In the preliminary NJ analysis of each region, Modeltest selected the Tamura-Nei model (Tamura and Nei 1993) and the HKY85 model (Hasegawa et al. 1985) for the *COI* and *cytB* regions, respectively. These analyses showed the existence of two distinct clades in the haplotypes of *P. typicus* for each region (results not shown), and the haplotypes composing each clade were identical between the analyses of these regions. Therefore, these regions were assumed to have shared the common evolutionary process and thus all data sets were combined into a single matrix and it was analyzed simultaneously to achieve high resolution of phylogenetic relationships of *P. typicus*.

Combining haplotypes of the two genes, 25 haplotypes were recognized (see Table 1). For combined data of the *COI* and *cytB* regions, Modeltest selected the HKY85 model by the hierarchical likelihood test. Heuristic parameter settings were as follows: empirical base frequencies were A = 0.3300, C = 0.1546, G = 0.1656, and T=0.3498; transition/transversion ratio = 2.2784 (kappa = 5.2384); -ln L (unconstrained) = 1594.45565. The total number of rearrangements tried was 88463, and the score (-ln) of the selected tree was 1705.6616.

The ML tree showed the existence of two distinct clades in the haplotypes of *P. typicus*, both of which were supported by high (>95%) bootstrap values (Figure 2). The number of base substitutions between the two clades was 1.9% (14 out of 751, Table 2). Within-group genetic diversity was not significantly

different between these clades (*COI*: clade I 0.0016 ± 0.0005, II 0.0018 ± 0.0008; *cytB* I 0.0044 ± 0.0022, II 0.0026 ± 0.0019, Tamura-Nei method). Clade I consisted of 19 samples

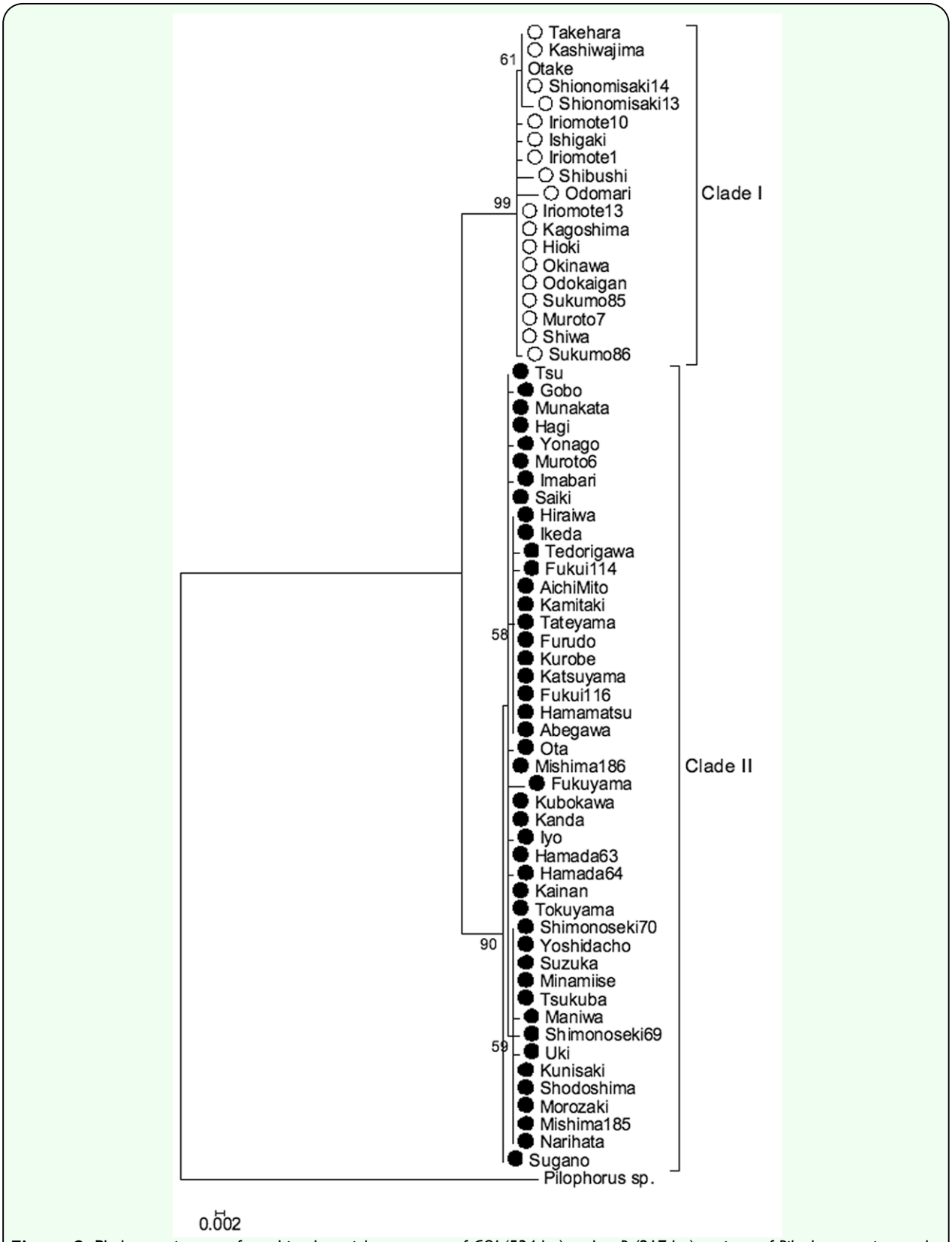

Figure 2. Phylogenetic tree of combined partial sequences of *COI* (534 bp) and *cytB* (217 bp) regions of *Pilophorus typicus* and an outgroup obtained by ML method. Numerals above the branches indicate bootstrap values (>50%, 1000 replicates). Black and white circles correspond with plots in Figure 3. High quality figures are available online.

(representing 9 haplotypes) that were found from 14 localities in the southern part of the range of *P. typicus* in Japan, i.e. the Ryukyus and the Pacific coastal parts of Kyushu, Shikoku, and Kinki districts with a few exceptional localities (Otake and Takehara) along the coast of the Seto Inland Sea (Figure 3). On the other hand, Clade II consisted of 45 samples (16 haplotypes) from 41 localities in the northern part of its range: from northern Kyushu to the central part of Honshu through northern Shikoku and most parts of Chugoku and Kinki (Figure 3). Of the 8 localities where 2 or 3 individuals were sampled, 7 localities were represented by either Clades I or II, and one locality in the southern Shikoku (Muroto) included both Clades I and II haplotypes. Considering that both types exist in only a few samples, localities in which both types reside may be more than shown in this result. These results suggest that the two clades have different distribution ranges (Figure 3), but in southwestern parts of Japan individuals of both groups are living sympatrically.

The observed distribution of the two clades suggests discordance between variation in DNA sequences and previously reported morphological variation. A previous study has revealed the existence of two morphologically distinct forms, recognized by a different structure of male genitalia, in *P. typicus* by a broad sampling from East and Southeast Asia including Japan, Taiwan, Malaysia, and Indonesia (Nakatani Y unpublished data; Yamada K, personal communication). However, to date, separation of their distribution ranges has been found only between Ishigaki and Iriomote Islands and no other morphological delimitation within the

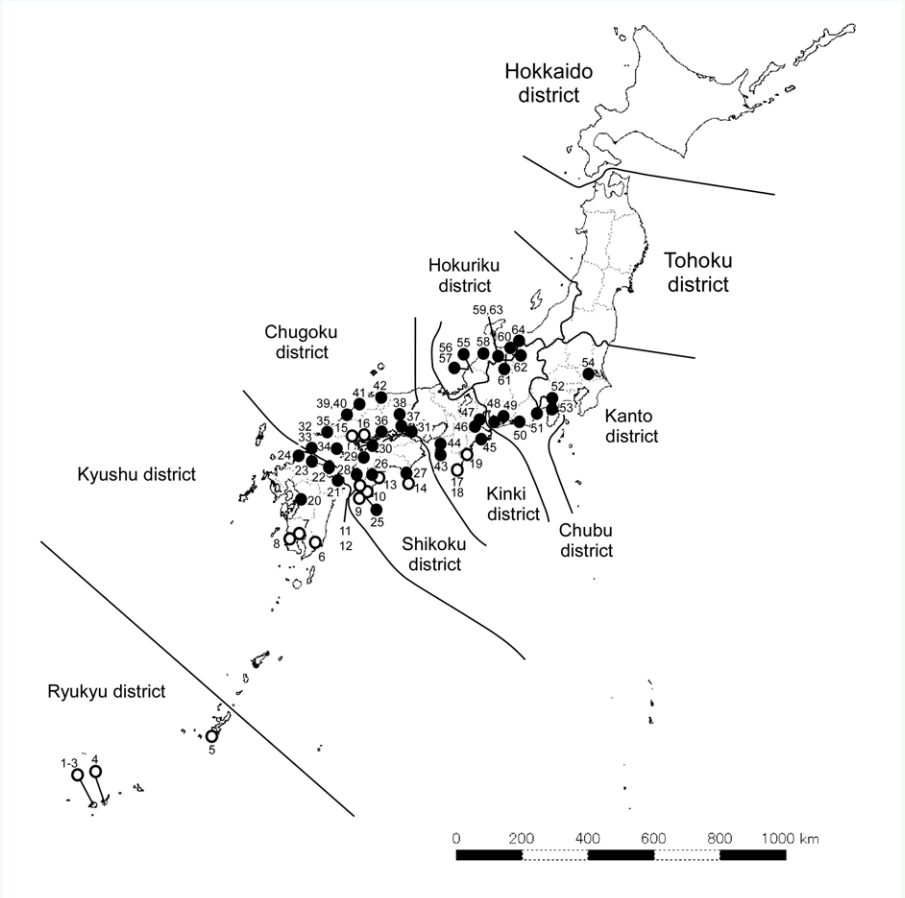

Figure 3. Geographical distribution of the two clades of *COI + Cytb* haplotypes of *Pilophorus typicus* in Japan. Open and closed circles represent Clade I and II, respectively. Numbers correspond to populations in Table 1. High quality figures are available online.

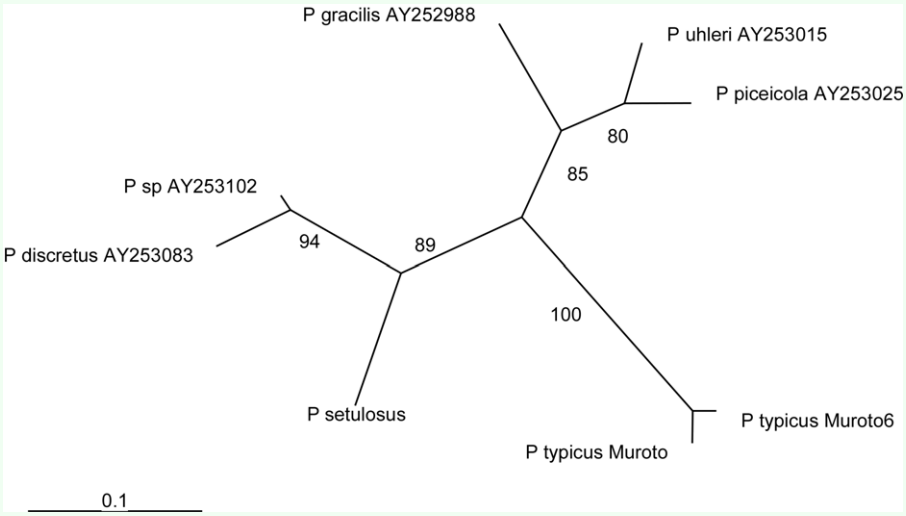

Figure 4. Unrooted tree of partial sequences of *COI* (533 bp) of *Pilophorus typicus* and congeneric species. Numerals above the branches indicate bootstrap values (>50%, 1000 replicates). High quality figures are available online.

Japan archipelago. Therefore, it is possible that genitalia structures could change within a short evolutionary period in which mitochondrial DNA sequences scarcely vary.

Phylogenetic analysis incorporating other species of *Pilophorus* revealed that genetic difference between the two groups was small at the intrageneric level, and thus suggest that they may have been differentiated only recently (Figure 4: GTR+G model; base frequency A = 0.3358, C = 0.1765, G = 0.1630, T = 0.3247; gamma shape parameter = 0.2212; -ln L (unconstrained) = 1594.98329; No. rearrangements = 180; Score of best tree = 1974.17678). Though this phylogenetic proximity does not immediately reflect the degree of reproductive isolation (e.g. Palumbi and Metz 1991), phylogenetically close populations may tend to hybridize more easily than distant ones (cf. Coyne and Orr 1997; Tubaro and Lijtmaer 2002). Therefore, scrutiny of reproductive isolation between the two groups should be investigated to infer the possible risks of disturbing the genetic structures of local populations through genetic introgression. Moreover, introducing genetically different strains may disturb the environment through secondarily damaging nontarget insects (Howarth 1991; Simberloff and Stiling 1996). Hybridization might enhance this process since it serves as a source of new variation. Before introducing *P. typicus* as a biological control agent for crop pests, the details of their ecological aspects such as potential host preference of these two groups and their reproductive compatibility should be adequately investigated, and the genetic and ecological impacts on the agroecosystem of application sites should be assessed.

Acknowledgements

We wish to thank Dr K. Miura for helpful advice on our experiments; Y. Hamachi, Y. Sugata, C. Araki, T. Kimura, K. Nakayama, M. Takeuchi, R. Kashitani, T. Kawabata, and Y. Ikemoto for experimental assistance and sample collection; Dr. S. Gale for reviewing the manuscript; Dr. K. Yamada for taxonomic information of this species; Drs. K. Yoshizawa and J. Yokoyama for helpful comments on phylogenetic analyses. This study was supported by Japan Science and Technology Agency (JST).

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